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Published in:
Biochemical and Biophysical Research Communications

DOI:
10.1016/S0006-291X(88)80908-2

Publication date:
1988

Vol. 156, No. 2, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1988 Pages 757-761

IMMUNOLOGICAL DETECTION OF G-PROTEIN α-SUBUNITS IN *DICTYOSTELIUM DISCOIDEUM*

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Received August 22, 1988

SUMMARY: Putative G-protein α-subunits in *Dictyostelium discoideum* were detected on western blots using the antiserum A-569, raised against a peptide whose sequence is found in α-subunits of all known GTP-binding signal transducing proteins. Two bands with a M_w of 40 kDa and 52 kDa were specifically recognized by the common peptide antiserum; the staining of both bands was strongly reduced when the antiserum was preincubated with the peptide that was used for antibody production. *D.discoideum* mutant HC213 (*fgd* A) lacks staining of the 40 kDa band, while the 52 kDa band is still present. This mutant is severely defective in cAMP receptor-G-protein interaction. We concluded that the primitive eukaryote *D.discoideum* contains proteins which show functional and physical similarity with the α-subunits of vertebrate G-proteins.

Transmembrane signal-transduction in *D.discoideum* may serve as a model for ligand-induced chemotaxis and cell differentiation in lower eukaryotes. Extracellular cAMP binds to specific surface receptors (1), and induces many biochemical and physiological responses. These include the alteration of the extracellular concentrations of protons, calcium ions and potassium ions, the activation of adenylate cyclase, guanylate cyclase (see references 2,3 for reviews) and phospholipase C (4), and the phosphorylation of cAMP receptors (5), myosin heavy and light chains (6). *D.discoideum* could be especially valuable for the study of transmembrane signal transduction because of its relatively small haploid genome, which allows the isolation and characterization of chemosensory mutants.

Several investigations suggest that the surface cAMP receptor is coupled to intracellular effector enzymes via G-proteins. It has been shown that cAMP binding to membranes is altered by guanine nucleotides (7) and, alternatively, cAMP alters GTP binding (8,9). In addition, cAMP stimulates high affinity GTP-ase activity in *D.discoideum* membranes (unpublished observations). Furthermore, *D.discoideum* adenylate cyclase is stimulated (10) and inhibited (11) by GTP, depending on the conditions used. Inhibition of adenylate cyclase by GTP is blocked by pretreatment of cells with pertussis toxin. Finally, GTP stimulates IP_3 formation in permeabilized cells (4). Although these investigations strongly suggest the functional interaction of surface receptors with effector enzymes via G-protein(s), no physical evidence for G-proteins in *D.discoideum* has been presented.

0006-291X/88 $1.50

We have used antisera against vertebrate G-proteins to detect *D.discoideum* proteins on western blots. Serum A-569 (12), which is directed against a peptide sequence of the putative GTP-binding site that is nearly identical in all known G-proteins, specifically detects two bands with an apparent M_w of 40 and 52 kDa. Furthermore, the 40 kDa band is strongly reduced in a mutant which is defective in receptor-G-protein interactions.

MATERIALS AND METHODS

Materials. [^{125}I]-protein A (2-10 μCi/μg) was from NEN, bovine serum albumin (BSA) was from Sigma, nitrocellulose was from Schleiger and Schuell. Antiserum A-569 was a generous gift of Dr. S. Mumby (12).

Culture conditions. *Dictyostelium discoideum*, NC-4(H), and mutant strain HC-213 (kindly provided by Dr. Barry Coukell, York University, Toronto) were grown in association with *Escherichia coli* on buffered glucose peptone agar. Cells were harvested in 10 mM KH_2PO_4/Na_2PO_4, pH 6.5 (PB), washed by repeated centrifugations for 2 min at 100 x g, and resuspended in this buffer at a density of 10^7 cells/ml.

Protein isolation and western blots. Cells were shaken in this buffer for 5 h, washed twice in PB, once in buffer A without sucrose, and the pellet was resuspended in buffer A (40 mM Hepes, 0.5 mM EDTA, 250 mM sucrose, pH 7.2). Cells were lysed at a density of 1.5 x 10^8 cells/ml by pressing them through a Nucleopore filter (pore size 3 μm). The homogenate was centrifuged for 5 min at 10,000 x g in an Eppendorf centrifuge; the supernatant was removed, and the pellet was washed once in buffer A. In some experiments 100 μl of the homogenate were centrifuged for 30 min at 160,000 x g in a Beckman Airfuge. The supernatants and the final pellets were made to the equivalent of 10^8 cells/ml in sample buffer (13); the supernatant contained 2.5 mg/ml soluble protein and the pellet fraction contained 0.8 mg/ml crude membrane protein, 60 μl of both fraction were applied to a 10% SDS polyacrylamide gel (14). The gel was run for 16 h at 40 mV and proteins were transferred to nitrocellulose (15). Nitrocellulose was incubated for 30 min in block buffer (6% BSA in wash buffer), and 60 min with the first antibody (200-fold dilution of serum A-569 in block buffer). Nitrocellulose was then washed 5 times for 10 min with wash buffer (16), incubated for 30 min with [^{125}I]-protein A (10 μCi/ml in wash buffer), washed 5 times for 10 min with wash buffer, air dried and autoradiographed for about 2 days using Kodak X-omat AR film and one intensifying screen.

In some experiments the antiserum was preadsorbed with the peptide that was used for its production (12). Briefly, 25 μl serum were incubated for 16 h at 6^{o}C with 62.5 μg of peptide CGAGESGKSTIVKQMK in a total volume of 125 μl. The serum + peptide was diluted to 5 ml with wash buffer and used further as described above.

RESULTS AND DISCUSSION

The aim of the present study was to obtain physical evidence for the existence of G-proteins in *D.discoideum*. Several approaches have not been successful. Photoaffinity labelling of receptor coupled G-proteins is hindered by the low affinity of 8-N_3-GTP-binding to *D.discoideum* membranes (9). Cholera-toxin does not alter signal transduction in *D.discoideum* and ADP-ribosylation experiments *in vitro* yield results which are difficult to interpret (unpublished observations). Pertussis toxin treatment does induce a strong and specific phenotype *in vivo* which is still detectable in isolated membranes (11). However, we have not been able to find conditions for pertussis toxin-specific ADP-ribosylation of membrane proteins *in vitro*. We report here on the detection of two *D.discoideum* membrane proteins using antisera which react specifically with G-protein α-subunits in vertebrates.

The antibody used was generated against a peptide that represents one of the parts of the GTP-binding site that is nearly identical in all α-subunits of G-proteins sequenced thus far. A western blot of size-separated *D.discoideum* proteins stained with this antibody is shown in Fig. 1. Two predominant bands were detectable with apparent M_w of 40 and 52 kDa (Fig. 1A). The antigens are present in the soluble and membrane fractions of a cell homogenate which was centrifuged for 5 min at 10,000 x g; a similar distribution was obtained when the homogenate was centrifuged for 30 min at 160,000 x g (data not shown). The specificity of the antiserum was evaluated by preadsorbing the antibody with the peptide that was used to generate the G-protein antibody. The results show that staining of the 40 and 52 kDa bands did not appear in membranes when the antiserum was preincubated with the peptide (Fig. 1B). In the soluble fraction preincubated with the peptide staining of 40 and 52 kDa bands was greatly reduced while the intensity of the nonspecific bands throughout the lane was not changed.

A class of *D.discoideum* mutants (*fgd* A) show a strongly altered interaction between cAMP-receptor and a putative G-protein (Table I; 17,18). cAMP is not able to induce chemotaxis or the accumulation of intracellular cGMP and cAMP levels in mutant cells. The major biochemical defects are a reduced inhibition by GTPγS and GDPβS of cAMP-binding, and a reduced stimulation by (Sp)-cAMPS of GTPγS binding to membranes. A western blot of *fgd* A mutant HC 213 is shown in Fig. 2. The staining of wild-type material with the G-

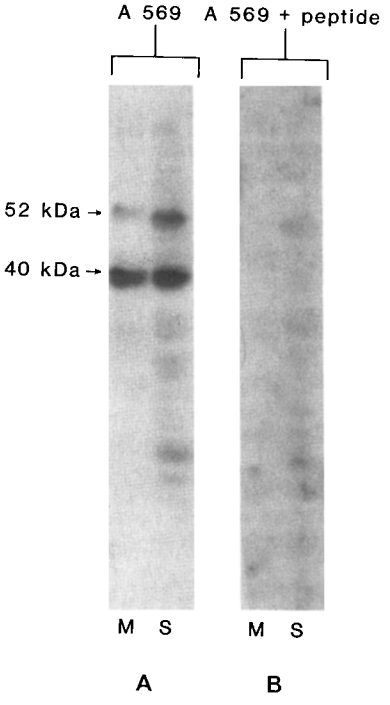

Figure 1 Western blot of *D.discoideum* proteins with common G_α antiserum.
Membrane (M) and soluble (S) proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with antiserum A-569 that detects multiple α-subunits of vertebrate G-proteins. A, antiserum A-569; B, antiserum A-569 preincubated with the peptide that was used for the generation of the antiserum.

Table I Signal Transduction in *fgd* A Mutant

Experiments	Wild-type	HC 213
cAMP-induced chemotaxis	100%	< 5
cGMP response	100%	< 5
cAMP response	100%	< 5
cAMP-binding	100%	35 ± 15
% inhibition by GTPγS	65 ± 12	36 ± 5
% inhibition by GDPβS	45 ± 11	30 ± 8
GTPγS-binding	100%	102 ± 5
% stimulation by cAMPS	27 ± 4	8 ± 7

The assay for chemotaxis (19), cGMP response (20), cAMP response (21), cAMP binding (7), were performed as described. The assay for GTPγS binding was similar to that for cAMP binding and contained 0.1 nM [^{35}S]GTPγS with or without 10 μM (Sp)-cAMPS (9). A more extensive description of signal-transduction in four *fgd* A mutants will be presented elsewhere (18).

protein antiserum is similar to that shown in Fig. 1A with 40 and 52 kDa bands in the soluble and membrane fraction. The mutant clearly shows staining of the 52 kDa band, which is, however, less intense than in wild-type material. In contrast, no specific staining was detectable at the position of the 40 kDa band, in either the soluble or membrane fraction. Staining present in the soluble fraction of the mutant was caused by staining of bands closed to the 40 kDa band, which were also present in wild-type supernatant. This western blot also demonstrates the staining of α-subunit of transducin (M_w = 39 kDa) by the G-protein antiserum.

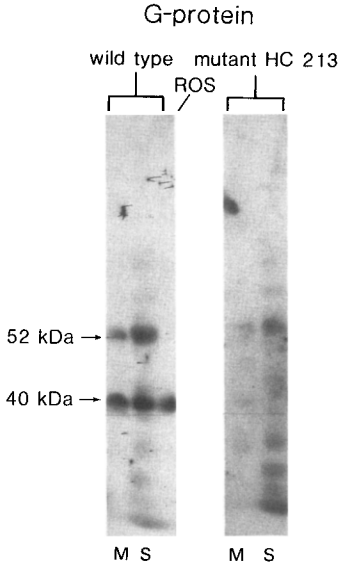

Figure 2 Western blot of *D.discoideum* proteins from mutant cells.
Membrane (M) and soluble (S) proteins from wild-type and mutant cells were probed with the G-protein antiserum. ROS is bovine rod outer segments (1 μg of protein) containing the α-subunit of transducin (M_w = 39 kDa).

The evolutionary distance between *D.discoideum* and vertebrates is billions of years. Therefore we used antisera which were directed against those parts of G-proteins which show the strongest homology between all G-protein α-subunits sequenced thus far. In *D.discoideum*, this antiserum detects two predominant bands with a M_w of 40 and 52 kDa. These results strongly suggest that the 40 and 52 kDa proteins are α-subunits of G-proteins. The functional identity of these putative G-proteins α-subunits is not yet resolved; based on the apparent molecular weight, it seems likely that the 52 kDa band could represent a G_s-like protein for which functional evidence has been presented (10,11). The 40 kDa band could be any α-subunit that belongs to the family of G_i-like proteins, including G_i and G_p, for which functional evidence has also been shown (11,4). *D.discoideum* may contain still other G-proteins that are detectable with the antiserum used in this study, and conversely, the antiserum may not detect all G-proteins that are present in *D.discoideum*.

The *fgd* A mutants will be very valuable for resolving the function of the 40 kDa protein. Not only is the interaction between receptor and putative G-protein altered in these mutants, but chemotaxis, and the activation of adenylate and guanylate cyclases are severely reduced *in vivo* (17). This suggests that the 40 kDa α-subunit of a putative G-protein fulfills a major function in *D.discoideum* signal transduction.

ACKNOWLEDGEMENTS

We gratefully acknowledge Susanne Mumby and Peter Devreotes for stimulating discussions. This work was supported by the Organisation for Fundamental Medical Research (Medigon) and the C. and C. Huygens Fund, which are subsidized by the Netherlands Organisation for the Advancement of Scientific Research (NWO).

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